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Phylogeographical and cytogeographical history of *Artemisia herba-alba* (Asteraceae) in the Iberian Peninsula and North Africa: mirrored intricate patterns on both sides of the Mediterranean Sea

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Artemisia herba-alba is an important component of Mediterranean dry steppe floras, being widely distributed in arid areas of the Iberian Peninsula and North-West Africa. In this study, we use genetic, cytogenetic and niche modelling tools to investigate the natural history of the species, focusing particularly on the role played by polyploidization to explain current diversity patterns throughout the main distribution range of the plant. Our sequencing data indicate a complex phylogeographical structure showing similar haplotype diversity patterns on both sides of the Strait of Gibraltar and no clear signals of genetic refugia. According to our cytogeographical results, we inferred multiple polyploidization events, which probably took place on the Iberian Peninsula and in North Africa independently. Environmental niche modelling suggested stable potential distributions of *A. herba-alba* on both sides of the Mediterranean Sea under present and past Last Glacial Maximum conditions, which could be related to the intricate spatial genetic and cytogenetic patterns shown by the species. Finally, environmental modelling comparison among cytotypes revealed that the niche of tetraploids is narrower and nested in that of diploids, a result that could indicate environmental specialization and could potentially explain recurrent establishment success of tetraploids.

ADDITIONAL KEYWORDS: autopolyploidy – diploids – flow cytometry – Iberian Peninsula – niche modelling – North-West Africa – plastid DNA – polyploidy – tetraploids.

INTRODUCTION

The Mediterranean region is considered one of the 25 world biodiversity hotspots, especially given its

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high plant species richness and level of endemism (Myers *et al.*, 2000). Consequently, the Mediterranean Basin has long attracted the attention of biologists interested in understanding the diversification processes of plants (Médail & Diadema, 2009). Remarkable phylogeographical patterns explaining the outstanding diversity of the region and neighbouring areas have principally been reported for species from the Mediterranean islands and southern European peninsulas (Weiss & Ferrand, 2007). In contrast, studies on taxa distributed in North Africa are much scarcer, and the evolutionary histories of these taxa have been considerably less deeply explored to date (Nieto Feliner, 2014; Médail & Baumel, 2018). The still limited phylogeographical research of North African plants has mainly focused on the Strait of Gibraltar, emphasizing the importance of this biogeographical area in understanding the emergence and maintenance of the rich Mediterranean biota (Hewitt, 2011). Intrinsic characteristics of this region such as the heterogeneous relief, the Strait as a temporally changing geographical barrier or a spatially diverse but locally stable climate have been inferred as key features to explain the high degree of genetic and taxonomic distinctiveness found in the Mediterranean area (e.g. Jaramillo-Correa *et al.*, 2010; García-Aloy *et al.*, 2017; Herrando-Moraira *et al.*, 2017; Massó, López-Pujol & Vilatersana, 2018). These geographical and climatic factors would have facilitated processes including vicariance, long-distance dispersal, hybridization and ecological adaptation (Thompson, 2005), jointly shaping the current plant diversity of North Africa and southern Europe. However, phylogeographical studies focusing on widespread plant species distributed on both sides of the Mediterranean Basin are still limited [for recent examples with representative sampling from African and European distribution ranges, see Magri *et al.* (2007) in *Quercus suber* L.; Guzmán *et al.* (2017) in *Chamaerops humilis* L.; Villa-Machío *et al.* (2018) in *Lavatera maritima* Gouan], and other potentially important evolutionary processes could have been overlooked as a result.

Polyploidy is recognized as one of the major evolutionary forces driving plant diversification by promoting adaptation to new ecological niches or conferring reproductive isolation (Otto & Whitton, 2000). In the Mediterranean region, examples of species experiencing polyploidization are numerous (Marques *et al.*, 2018), and a few of them have been thoroughly explored from taxonomic, phylogenetic and ecological points of view (e.g. Jakob, Ihlow & Blattner, 2007; Balao *et al.*, 2010; Bardy *et al.*, 2010; Zozomová-Lihová, Marhold & Španiel, 2014) and these studies emphasized the importance of auto- and allopolyploidization in the richness of the Mediterranean flora. Biogeographical processes, including historical patterns of origin or migration, interactions among cytotypes and divergence in levels of environmental tolerance have typically been reported as the main factors determining the success of populations with different ploidies (Husband, Baldwin & Suda, 2013). However, the mechanisms of establishment and spread of polyploid complexes in the Mediterranean region are still poorly known (Marques *et al.*, 2018). In this sense, more studies combining cytogeographical information, phylogeographical data and environmental niche modelling (ENM) in mixed-ploidy species are necessary to better understand the role of whole-genome multiplications in Mediterranean plant diversity.

Artemisia herba-alba Asso (Asteraceae, Anthemideae) belongs to *Artemisia* L. subgenus *Seriphidium* (Besser ex Less.) Rouy and it has been referred to as a species complex from the Mediterranean region, with closely related taxa considered as independent species in the Irano-Turanian region, or as a single species (e.g. Vallès, 1987; Ouyahya & Viano, 1988; Vallès *et al.*, 2011; Podlech, 2013; Bougoutaia *et al.*, 2014). Recent phylogenetic studies (e.g. Malik *et al.*, 2017) indicate that *A. herba-alba s.s.* would better constitute a single species basically distributed in the Iberian Peninsula and North-West Africa, whereas other Irano-Turanian taxa formerly included in the complex (e.g. *A. inculta* Delile, *A. oliveriana* J.Gay ex Besser and *A. sieberi* Besser; often treated as synonyms of *A. herba-alba*) were inferred to be evolutionarily distant from *A. herba-alba* and should be considered as separate species. This perennial small shrub is an important component of Mediterranean dry steppe floras, being the main forage species in chamaephytic steppes of North Africa, where it covers *c.* 10 million ha (El Aich, 1992; Le Houérou, 2001). Consequently, *A. herba-alba* has been well studied from ecophysiological and grazing management perspectives (e.g. Escudero *et al.*, 2000; Houmani, Houmani & Skoula, 2004). The species has also been commonly used in folk medicine, mainly in North Africa, and the biochemical diversity and activity of the plant have also been widely explored (Mighri *et al.*, 2010; Mohamed *et al.*, 2010; Younsi *et al.*, 2018). From an evolutionary point of view, a few studies have focused on the cytogenetic diversity of *A. herba-alba* (e.g. Vallès, 1987; Ferchichi, 1997; Torrell & Vallès, 2001; Torrell *et al.*, 2003; Betina, Khalfallah & Khelifi, 2007; Bougoutaia *et al.*, 2014, 2016), revealing the existence of two ploidies in the species in both European and North African populations. The distribution of diploid and polyploid (tetraploid) cytotypes of *A. herba-alba* in Algeria was explained as resulting from a process of genome differentiation,

which could be related to environmental and biogeographical factors (Bougoutaia *et al.*, 2016), but the limited sampling and missing phylogeographical context hampered further inferences on the role of ploidy during the evolutionary history of the species.

The main objective of this study is to obtain a deeper and detailed knowledge of the natural history of *A. herba-alba* as a key species of dry steppes from the Iberian Peninsula and North Africa. We hypothesize that the Strait of Gibraltar played an important role on shaping the genetic diversity of this species. Given the occurrence of distinct cytotypes, we also expect to find a genetic structure associated with ploidies, which could illuminate the origins of whole genome duplication in *A. herba-alba*. Finally, we suggest that certain ecological differentiation among diploid and polyploid cytotypes could have driven the establishment and spread of this species in the western Mediterranean region. To test these hypotheses, we applied a multidisciplinary approach combining spatial genetic and cytogenetic analyses with ENM. Specifically, we: (1) used flow cytometry to assess the genome size and infer the ploidy of 185 individuals of *A. herba-alba* on both sides of the Mediterranean Sea; (2) reconstructed a phylogeographical framework for this species from 388 sequences of plastid DNA regions, which have been argued as particularly useful markers when investigating heteroploid plant systems (Záveská *et al.*, 2019); and (3) performed species distribution modelling, under present and past climatic scenarios, and calculated environmental differences between cytotypes.

MATERIAL AND METHODS

Sampling, genome size estimates and DNA sequencing

Forty populations of *A. herba-alba* were sampled, covering the main distribution range of the species on the Iberian Peninsula (12 populations) and in North Africa (28 populations). Leaf material from five to ten plants per population was collected and stored fresh (for genome size assessment) and dried in silica gel (for DNA sequencing procedures). Voucher specimens were deposited at the herbarium BCN, of the Centre de Documentació de Biodiversitat Vegetal (Universitat de Barcelona). To minimize excessive sampling from within the progeny of a single maternal plant, the individual samples were collected from plants at least 10 m apart. Further details about the studied material (population origin, geographical coordinates, number of analysed individuals and herbarium vouchers) are given in Table 1 and in the Supporting Information (Table S1).

The genome size of 39 populations was estimated by flow cytometry at the Centres Científics i Tecnològics, Universitat de Barcelona (CCiTUB), following the procedures explained by Bougoutaia *et al.* (2016). Five individuals were analysed in most populations, except for P40 (four individuals), P19, P38, P45 (three individuals) and P48 (two individuals) due to availability of fresh material. Two independent replicates of each individual were performed. The analyses for a given population were all performed on the same day. *Petunia hybrida* Vilm. 'PxPc6' and *Pisum sativum* L. 'Express Long' (Marie & Brown, 1993) were used as internal standards. Seeds of the standards were provided by the Plateforme de cytométrie d'Imagerie-Gif, CNRS – I2BC (Gif-sur-Yvette, France). Nuclear DNA contents (2C) were calculated by multiplying the known DNA content of the standard by the quotient between the peak positions (mode) of the target species and the standard in the histogram of fluorescence intensities, assuming a linear correlation between the fluorescence signals from the stained nuclei of the unknown specimen, the known internal standard and the DNA amount (Doležel, 1991). The genome size values of 12 populations from Algeria (see Supporting Information, Table S1) were obtained from Bougoutaia *et al.* (2016).

Leaf tissue dried in silica gel (*c.* 20 mg) was used for DNA extraction using the CTAB protocol (Doyle & Doyle, 1987) with minor modifications. The quality and quantity of DNA extracts were checked with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The plastid intergenic regions *rpl32-trnL* and *ndhC-trnV* were amplified and sequenced for all samples. The amplification procedure was performed as described by Malik *et al.* (2017). Direct sequencing of the amplified DNA segments was performed with Big Dye Terminator Cycle Sequencing v.3.1 (PE Biosystems, Foster City, CA, USA) at the Unitat de Genòmica (CCiTUB) on an ABI PRISM 3700 DNA analyser (PE Biosystems). The sequencing primers used were the same as those for amplification. Sequences were edited and assembled using Chromas Lite v.2.01 (Technelysium PTy, Tewantin, Queensland, Australia) and Bioedit v.7.0.9 (Ibis Biosciences, Carlsbad, CA, USA). The alignment was conducted in Clustal W (Thompson, Higgins & Gibson, 1994) and adjusted manually. GenBank accession numbers are provided in the Supporting Information (Table S1).

Genetic analyses

Plastid haplotypes were determined from nucleotide substitutions in a combined data set that included both the *rpl32-trnL* and the *ndhC-trnV* regions. Gaps resulting from indels and mononucleotide repeat

Table 1. Sampling information, estimated ploidy and haplotypes (*Hd*, haplotype diversity; π, nucleotide diversity) of studied *Artemisia herba-alba* populations

Population code*	Collection data	*N*	Ploidy†	Haplotype(s)	*Hd*	π
P3	Algeria, Djelfa: Ben-Hamed	5	4*x*	H1(5)	0	0
P4	Algeria, B. B. Arreridj: El-Euch	5	4*x*	H1(5)	0	0
P6	Algeria, M'sila: Zerarka	5	2*x*/4*x*	H1(2), H2(3)	0.600	0.0004
P7	Algeria, M'sila: Mohamed Boudiaf	5	2*x*	H1(5)	0	0
P9	Algeria, M'sila: Ouled Slimane	4	2*x*	H1(2), H2(1), H3(1)	0.833	0.0007
P11	Algeria, Laghouat: Sebgag	5	4*x*	H1(4), H4(1)	0.400	0.0027
P12	Algeria, Sétif: Hammam soukhna	5	4*x*	H1(5)	0	0
P14	Algeria, Tiaret: Rechaiga	4	4*x*	H1(4)	0	0
P15	Algeria, Tiaret: Ain Dheb	5	4*x*	H1(5)	0	0
P17	Algeria, Biskra: Baniane	5	2*x*	H2(1), H5(4)	0.400	0.0016
P19	Algeria, Batna: Arris	5	4*x*	H1(5)	0	0
P26	Algeria, Tébéssa: Oum Ali	5	4*x*	H1(2), H5(3)	0.600	0.0020
P31	Algeria, Souk-Ahras: Taoura	5	4*x*	H1(5)	0	0
P33	Algeria, Saida: Ain Skhouna	5	4*x*	H1(3), H6(2)	0.600	0.0004
P38	Algeria, S.B. Abbès: Marhoum	5	4*x*	H1(5)	0	0
P40	Algeria, Tlemcen: El-Aricha	4	4*x*	H1(4)	0	0
P60	Algeria, Tamanrasset: Tazrouk	7	2*x*	H19(7)	0	0
P42	Tunisia, Sidi Bouzid: Jemla	5	4*x*	H3(2), H5(2), H7(1)	0.800	0.0024
P43	Tunisia, Medenine: Neffatia	5	2*x*	H1(5)	0	0
P44	Tunisia, Medenine: IRA	5	–	H1(5)	0	0
P45	Tunisia, Medenine: Oued El Fedje	5	2*x*	H8(5)	0	0
P59	Morocco, Marrakaech: Imagdal	3	2*x*	H16(3)	0	0
P61	Morocco, Ouarzate: Taliouine	6	2*x*	H20(6)	0	0
P62	Morocco, Ouarzate: Skoura	5	4*x*	H4(3), H20(2)	0.600	0.0057
P63	Morocco, Midelt: Kerrandou	5	4*x*	H4(5)	0	0
P64	Morocco, Guercif: Outat El Haj	5	4*x*	H16(3), H21(1), H22(1)	0.700	0.0022
P65	Morocco, Nador: El Massira	5	4*x*	H16(2), H23(3)	0.600	0.0004
P66	Morocco, Midar: Tafersite	5	2*x*/4*x*	H16(2), H19(1), H24(2)	0.800	0.0009
P46	Spain, Aragon, Zaragoza: Bujaraloz	5	4*x*	H9(1), H10(1), H11(3)	0.700	0.0009
P47	Spain, Aragon, Zaragoza: Calatayud	5	4*x*	H9(4), H12(1)	0.400	0.0003
P48	Spain, Aragon, Zaragoza: Alhama de Aragón	2	4*x*	H9(2)	0	0
P49	Spain, Madrid: Aranjuez	5	2*x*	H13(1), H14(3), H15(1)	0.700	0.0027
P50	Spain, La Mancha, Ciudad Real: Argamasilla de Alba	5	2*x*	H13(3), H17(2)	0.600	0.0020
P51	Spain, Andalusia, Jaén: Carchelejo	5	2*x*	H13(3), H16(2)	0	0
P52	Spain, Andalusia, Granada: Cúllar	5	4*x*	H13(1), H16(1), H17(2), H18(1)	0.900	0.0031
P53	Spain, Andalusia, Almería: Níjar	5	4*x*	H16(5)	0	0
P54	Spain, Valencia, Alacant: Petrer	5	2*x*	H16(3), H17(2)	0.600	0.0004
P55	Spain, Valencia, Castelló: Sorita	5	2*x*	H9(5)	0	0

Table 1. Continued

Population code*	Collection data	*N*	Ploidy†	Haplotype(s)	*Hd*	π
P56	Spain, Catalonia, Barcelona: Castellfollit de Riubregós	5	2*x*	H10(4), H11(1)	0.800	0.0005
P57	Spain, Catalonia, Lleida: Arbeca	4	4*x*	H10(4)	0	0

*Population numbers are the same as in Figure 1.
†Ploidy (2*x*, diploid; 4*x*, tetraploid) inferred from genome size estimations (see Table S1 for genome size data).

units were treated as missing data. The evolutionary relationships among haplotypes were inferred based on a parsimony TCS network constructed using PopArt (Leigh & Bryant, 2015) with default settings. Haplotype (*Hp*) and nucleotide (*p*) diversities were calculated for each population using DnaSP v.5.0 (Rozas & Rozas, 1999). The same indices were estimated for groups of samples according to ploidy level (i.e. diploid and tetraploid populations) and geographical origin (i.e. Iberian and African populations). Haplotype richness [$R_{(n)}$] was computed with RAREFAC (Petit, el Mousadik & Pons, 1998), software that uses a rarefaction approach to standardize the haplotype richness to a fixed sample size to facilitate comparisons across groups of samples. In this case, the rarefaction value was set according to the sample size of the smallest groups of populations at ploidy and geographical levels (i.e. diploid group and Iberian group).

The molecular phylogenetic reconstruction of *A. herba-alba* haplotypes was performed by Bayesian inference with MrBayes v.3.2 (Ronquist *et al.*, 2012) based on the DNA sequences of the haplotypes obtained from the previous procedures. *Artemisia annua* L. and *A. chitralensis* Podlech were chosen as outgroups according to a phylogenetic study on *Artemisia* subgenus *Seriphidium* (Malik *et al.*, 2017). Partitioning strategies and models of molecular evolution were selected with PartitionFinder v.2.1.1 (Lanfear *et al.*, 2017). A scheme with two independent partitions (GTR model) was applied for both *rpl32-trnL* and *ndhC-trnV* intergenic spacers. Two independent Markov chain Monte Carlo (MCMC) analyses with four Metropolis coupled chains each were run for 10 million generations, sampling every 1000 generations. The first 25% of the trees were discarded as 'burn-in', after confirming that the average standard deviation of the split frequencies was < 0.01, and the potential scale reduction factor approached 1.0 for all parameters. The remaining trees were pooled to construct 50% majority-rule consensus trees that approximate the posterior distribution of the phylogenetic reconstructions and to obtain Bayesian posterior probabilities.

The existence of phylogeographical structure was tested by the permutation test between G_{ST} and N_{ST} (coefficients of genetic differentiation) implemented in PERMUT2.0 with 1000 permutations (Pons & Petit, 1996). The relationship between the genetic differentiation [*Dxy* Nei (1987), estimated through DnaSP] and the geographical distance per population pairs was determined through Mantel tests using three datasets: (1) all populations, (2) only Iberian populations and (3) only North African populations. Pairwise correlations between distance matrices were computed using 10 000 permutations with the function *mantel* available in the package 'vegan' (Oksanen *et al.*, 2019) of R v.3.5.2 (R Core Team, 2018). Haplotype spatial genetic structure was further analysed with SAMOVA2 (Dupanloup, Schneider & Excoffier, 2002), carrying out a simulated annealing approach to identify population clusters. We explored *K* values (i.e. numbers of groups of populations) from 2 to 20, starting from 100 random initial conditions for each simulation, and chose the number of groups that gave the highest ΔF_{CT} (i.e. F_{CT} differences between groups). Finally, we also conducted analysis of molecular variance (AMOVA) in Arlequin v.3.5 with 10 000 replicates (Excoffier & Lischer, 2010) to measure variation among populations and to test the genetic differentiation between groups of populations according to: (1) SAMOVA clustering, (2) the main geographical regions (i.e. Iberian Peninsula and North Africa) and (3) ploidy.

Ecological niche analyses

We performed ENM to analyse the potential distribution of *A. herba-alba* under present climatic conditions. We used MaxEnt v.3.3 (Phillips, Anderson & Schapire, 2006) software and employed the maximum entropy algorithm. Nineteen bioclimatic variables (at 30-s resolution) under current conditions and an elevation layer were obtained from the database of the WorldClim website (Fick & Hijmans, 2017) and clipped to cover the Iberian Peninsula and North Africa. After a combination of a correlation analysis

in a random sample of 1000 points within the study area plus jackknife and per cent contribution analyses to evaluate the relative importance of each variable, 11 relatively uncorrelated ($r < |0.85|$) variables were selected [bio1 (annual mean temperature); bio2 (mean diurnal range); bio3 (isothermality); bio4 (temperature seasonality); bio6 (minimum temperature of the coldest month); bio8 (mean temperature of the wettest quarter); bio9 (mean temperature of the driest quarter); bio12 (annual precipitation); bio15 (precipitation seasonality); bio18 (precipitation of the warmest quarter); and elevation]. Two soil variables (pH and organic content measured at 15 cm depth) were downloaded from ISRIC (World Soil Information; www.isric.org) and added to the dataset. These 13 variables were used together as predictors to calibrate the species distribution model. In the occurrence dataset, we employed the 40 georeferenced localities corresponding to the sampled populations of *A. herba-alba* (Fig. 1; Table 1). These occurrences were randomly split into training data (80%) and test data (20%), and 100 subsampled replicates were run for model evaluation, with the threshold obtained under the maximum training sensitivity plus specificity rule. The distribution model under current conditions was projected to the Last Glacial Maximum (LGM; *c.* 21 kyr BP) under two models: the community climate system model (CCSM; Collins *et al.*, 2006) and the model for interdisciplinary research on climate (MIROC; Watanabe *et al.*, 2010). Because no scenarios are available for the LGM performance of elevation and soil variables, they were discarded from the LGM projection models.

To compare the ecological niche between cytotypes, independent ENM analyses were performed for each subset of populations. In these cases, we used the same 13 variables and MaxEnt settings employed for the whole dataset under current climatic conditions. To calculate the differences on geographically suitable areas between cytotypes, the maximum sensitivity plus specificity (MSS) logistic threshold was used, a metric recommended as being robust with all data types (Liu, Newell & White, 2016); MSS was used as the 'cut-off' value to transform the continuous value outputs of MaxEnt to binary maps (absence/presence). Niche similarity between those groups of populations was assessed by estimating Hellinger-derived *I* and Schoener's *D* indices (Warren, Glor & Turelli, 2008) calculated with the niche overlap test implemented in the software ENMTools v.1.4.3 (Warren, Glor & Turelli, 2010). A test with 100 pseudo-replicates was calculated to generate a distribution of the expected values of each index. Histograms were constructed after performing both tests to visualize the niche differentiation. The differences on niche breadth of the different cytotypes was measured using the 'inverse concentration' B1 (Levins, 1968) and the 'uncertainty' B2 metrics in ENMTools, using 100 subsample iterations from MaxEnt to account for model uncertainty. Niche differences between diploid and tetraploid plants were also evaluated by a principal components analysis (PCA) approach using the same 13 variables. A three-dimensional environmental space for each group of plants was generated based on the observed occurrences defined by the first three axes that were identified by the PCA. Finally, we used Wilcoxon signed-rank and Levenne tests to explore the environmental variable differences (of medians and variances, respectively) between populations with different ploidies. These last statistical analyses (i.e. PCA, Wilcoxon signed-rank and Levenne tests) were performed in R v.3.5.2 (R Core Team, 2018) with the 'Rcmdr' package (Fox & Bouchet, 2020).

RESULTS

Cytogenetic and genetic data

Nuclear DNA amount data for the 39 studied populations (185 individuals) of *A. herba-alba* are presented in the Supporting Information (Table S1). Average nuclear DNA amounts ranged from 5.39 to 7.76 pg for diploid accessions and from 11.53 to 13.84 pg for tetraploids (Table S1). The relatively wide dispersion of genome size (GS) estimations within each ploidy could be due to genuine cytogenetic variation. For instance, the presence of B chromosomes has already been reported in *A. herba-alba* (Torrell *et al.*, 2003), potentially causing slight differences in nuclear DNA amount. However, although the coefficient of variation (CV) of the 2C peaks was always < 5%, we cannot be certain that technical issues (e.g. sample conservation) affected the precise GS estimation of certain populations. Therefore, we only employed the results of flow cytometry assessments to infer ploidy of specimens. These GS estimates revealed that 23 of the analysed populations contain only tetraploid plants and 14 only contain diploid plants. In two North African populations (P6 and P66), diploid and tetraploid individuals were intermixed. Diploid and tetraploid populations were present on both sides of the Mediterranean Sea, also showing a scattered geographical distribution in each continent (Fig. 1).

The sequences of the *rpl32-trnL* and *ndhC-trnV* intergenic spacers were aligned in two matrices containing 812 and 810 nucleotides, respectively. Both plastid DNA regions showed a noticeable level of polymorphism among the 194 specimens of *A. herba-alba* analysed in this study. Specifically, 27 and 11 polymorphic (segregating) sites were observed for

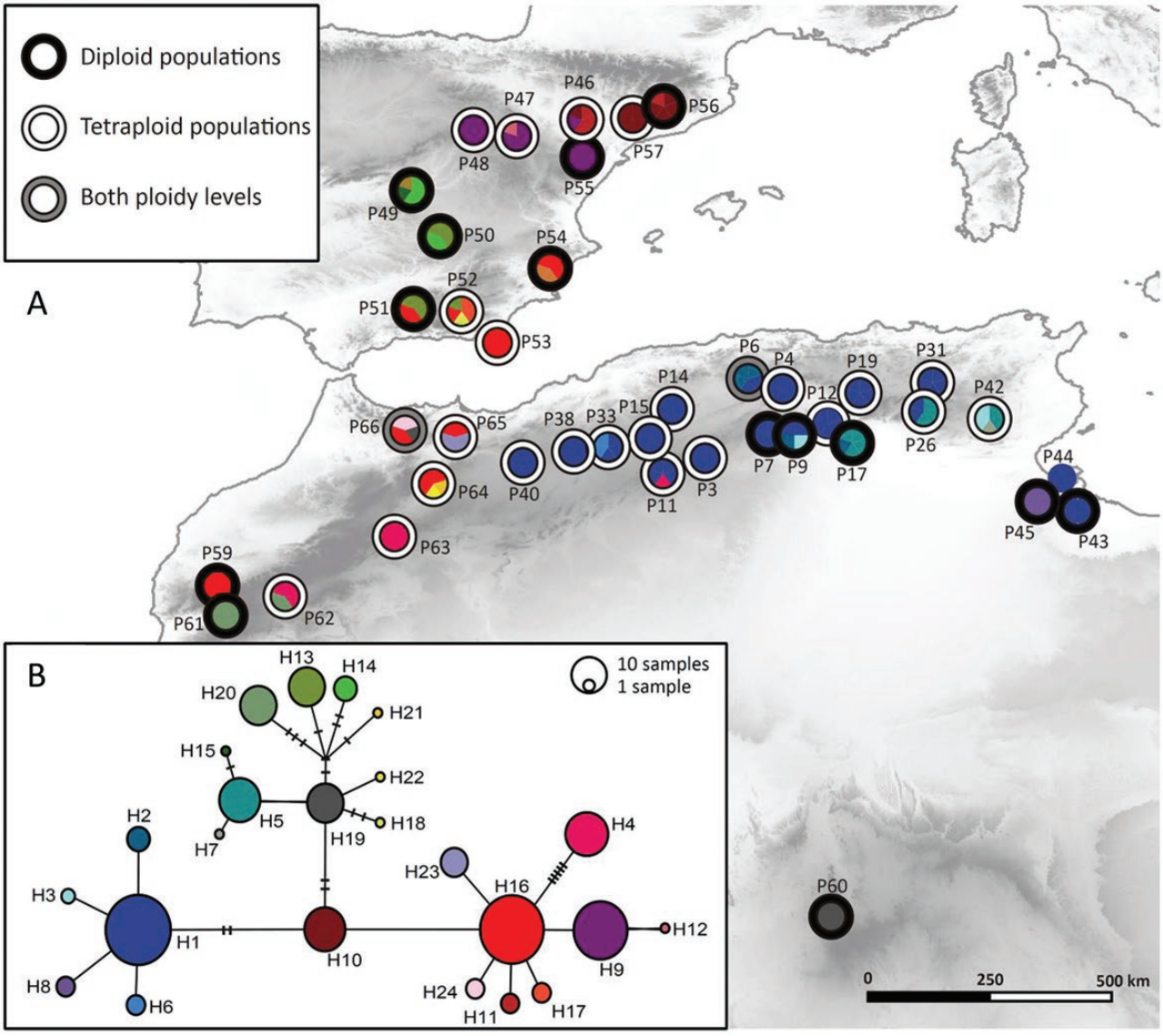

Figure 1. A, geographical distribution of the cytotypes and the plastid DNA haplotypes (see Table 1 for population codes) found in *Artemisia herba-alba*. B, statistical parsimony network of relationships between the haplotypes. Black bars represent unsampled intermediate haplotypes, one base mutation distant. The size of the circles represents the number of individuals. The ploidy of population P44 could not be assessed.

the *rpl32-trnL* and *ndhC-trnV* markers, respectively. Based on analysis of the concatenated matrix, we were able to identify 24 haplotypes across the 40 studied populations (Table 1). The TCS parsimony revealed a relatively complex evolutionary structure (Fig. 1), from frequent (e.g. H1; 71 individuals) to rare haplotypes (e.g. H7, H12, H15, H18, H21 and H22; one individual each) connected by one to six mutation steps. Only one haplotype (H16) was found both in the Iberian Peninsula and in North Africa; the rest occurred only in one of the regions (15 haplotypes in North Africa and ten on the Iberian Peninsula). However, haplotypes from different sides of the Strait of Gibraltar were intermixed according to the evolutionary relationships shown by the parsimony network (Supporting Information, Fig. S1). Regarding the ploidy of populations, the haplotype network was not structured according to cytotype distribution: most haplotypes (13) were shared by diploid, tetraploid and/or populations showing both ploidies (Fig. S2).

Half of the populations harboured only one haplotype, and the others showed different levels of haplotype and nucleotide diversity (Table 1). From a phylogeographical point of view, the samples from

Table 2. Genetic variability values for the geographical and cytogenetic groups of populations defined in the study

	No. of sampling sites	*N*	*Hp*	*Hd*	$R_{(n)}$	π
Iberian Peninsula	12	56	10	0.866	9.000	0.00237
North Africa	28	138	15	0.840	10.914	0.00265
Diploid populations	14	60	14	0.921	6.092	0.00325
Tetraploid populations	23	119	19	0.751	4.361	0.00262
Both ploidies	2	10	5	0.867	4.000	0.00218
All populations	40	194	24	0.836	–	0.00300

N, number of individuals; *Hp*, number of haplotypes; *Hd*, haplotype diversity; $R_{(n)}$, allelic richness after rarefaction; π, nucleotide diversity.

North Africa contained a greater number of haplotypes (15) than those from the Iberian Peninsula (10), but the latter showed higher genetic variability in terms of haplotype and nucleotide diversity (Table 2). Haplotype richness calculated after rarefaction [$R_{(56)}$] was higher in North Africa than on the Iberian Peninsula. Regarding the genetic variability among cytotypes, tetraploid populations contained more haplotypes (19) compared with diploid ones (14), but haplotype and nucleotide diversity, as well as haplotype richness [$R_{(10)}$] were higher in diploids than in tetraploids (Table 2). The phylogenetic reconstruction of *A. herba-alba* haplotypes (Fig. 2) inferred the existence of several strongly supported monophyletic lineages, most of them in derived positions of the tree. In contrast, early-diverging haplotypes were not grouped in statistically supported lineages. The phylogenetic tree did not cluster the haplotypes according to their geographical distribution or to the ploidy of populations.

The permutation test showed that haplotypes sampled from within populations are phylogenetically closer than haplotypes sampled from different populations (N_{ST} = 0.731, G_{ST} = 0.651; $P < 0.01$), indicating the existence of phylogeographical signal (Pons & Petit, 1996). The Mantel test found a significant correlation between the pairwise genetic differentiation and the geographical distance of populations from North Africa ($r = 0.4736$; $P < 0.005$), but not for the whole *A. herba-alba* dataset ($r = 0.1723$; $P > 0.005$) or for the Iberian populations ($r = 0.1284$; $P > 0.005$). Spatial genetic analyses of plastid DNA haplotypes using SAMOVA indicated that the largest increase of F_{CT} values occurred between $K = 2$ and $K = 3$ (Supporting Information, Fig. S3). The genetic structure shown by $K = 2$ clustered the populations from the Iberian Peninsula and Morocco plus two populations from Algeria (i.e. P17 and P26) in one group and the remaining populations from Algeria and Tunisian in another group (Fig. S4). The structure depicted by $K = 3$ showed one group constituted by Iberian and Moroccan populations, a second group with Iberian, Moroccan and Algerian populations, and a third cluster entirely constituted by the majority of populations from Algeria (Fig. S4). The results of AMOVAs studying the partitioning of genetic diversity are summarized in Table S2. Non-hierarchical AMOVA showed that 78.02% of the variation was explained by differences among populations, and 21.98% was explained by differences within populations. Hierarchical AMOVA according to the phylogeographical structure inferred by SAMOVA showed that genetic differences among $K = 2$ clusters explained 59.94% of the variance, and genetic differences among $K = 3$ clusters explained 71.23% of the variation. Running a hierarchical AMOVA in which the two main geographical regions were considered (North Africa and Iberian Peninsula) showed that differences among those groups represented 28.36% of the genetic variance. Hierarchical analysis with ploidy defining two groups of populations revealed that only 2.00% of the genetic variation was attributable to the between-cytotype component.

Ecological niche analyses

The distribution model of *A. herba-alba* under current conditions (Fig. 3) spanned its current distribution in the western Mediterranean Basin, including most sampled localities of the species. The mean area under the receiver operating characteristic curve (AUC; a measure of model fitness) for testing data was high (0.927), supporting the predictive power of the model. The standard deviation of the 100 replicates was low (0.005), and the omission rate using maximum training sensitivity plus specificity threshold was 4.44%. According to jackknife testing, the environmental variables with highest gain when used in isolation were bio12 (annual precipitation), bio1 (annual mean temperature) and the soil organic content, which therefore appeared to be the most informative (see Supporting Information). The environmental variable that reduced the gain the most when omitted was the pH of the soil, therefore appearing to have the most information that is not present in the other variables (see Supporting Information). The CCSM and MIROC models for the LGM yielded almost identical

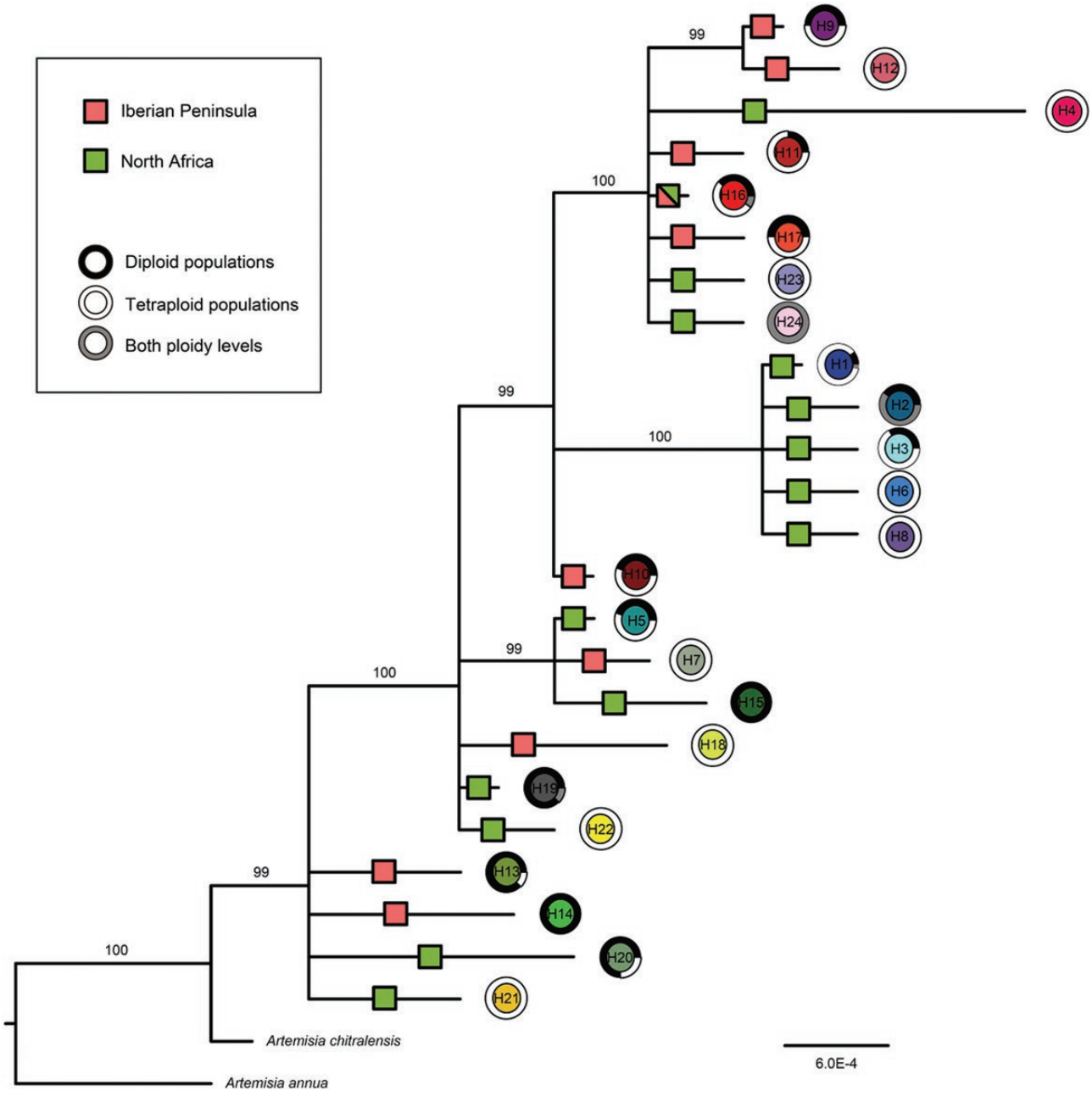

Figure 2. Phylogenetic tree of *Artemisia herba-alba* haplotypes based on Bayesian inference of the combined plastid regions. Posterior probability (PP) values are shown above branches. The colour of the squares placed over the terminal branches indicates the geographical distribution of the haplotypes. The rings in greyscale indicate the ploidy level of populations containing each haplotype.

inferences for the palaeodistribution of *A. herba-alba* as the model constructed using the same ten variables from the present (Fig. 3). In all cases, AUC values were reasonably high (> 0.80), the most important variables being bio12 (annual precipitation), bio6 (minimal temperature of coldest month) and bio18 (precipitation of warmest quarter). The two LGM models predicted only minor area changes (i.e. an increase of *c*. 7%) compared to the present potential distribution (Supporting Information, Table S3).

Independent ENM analyses predicted the potential current distribution ranges for each subset of populations showing different ploidies (Fig. 4A, B). Both models provided reasonably good estimates of

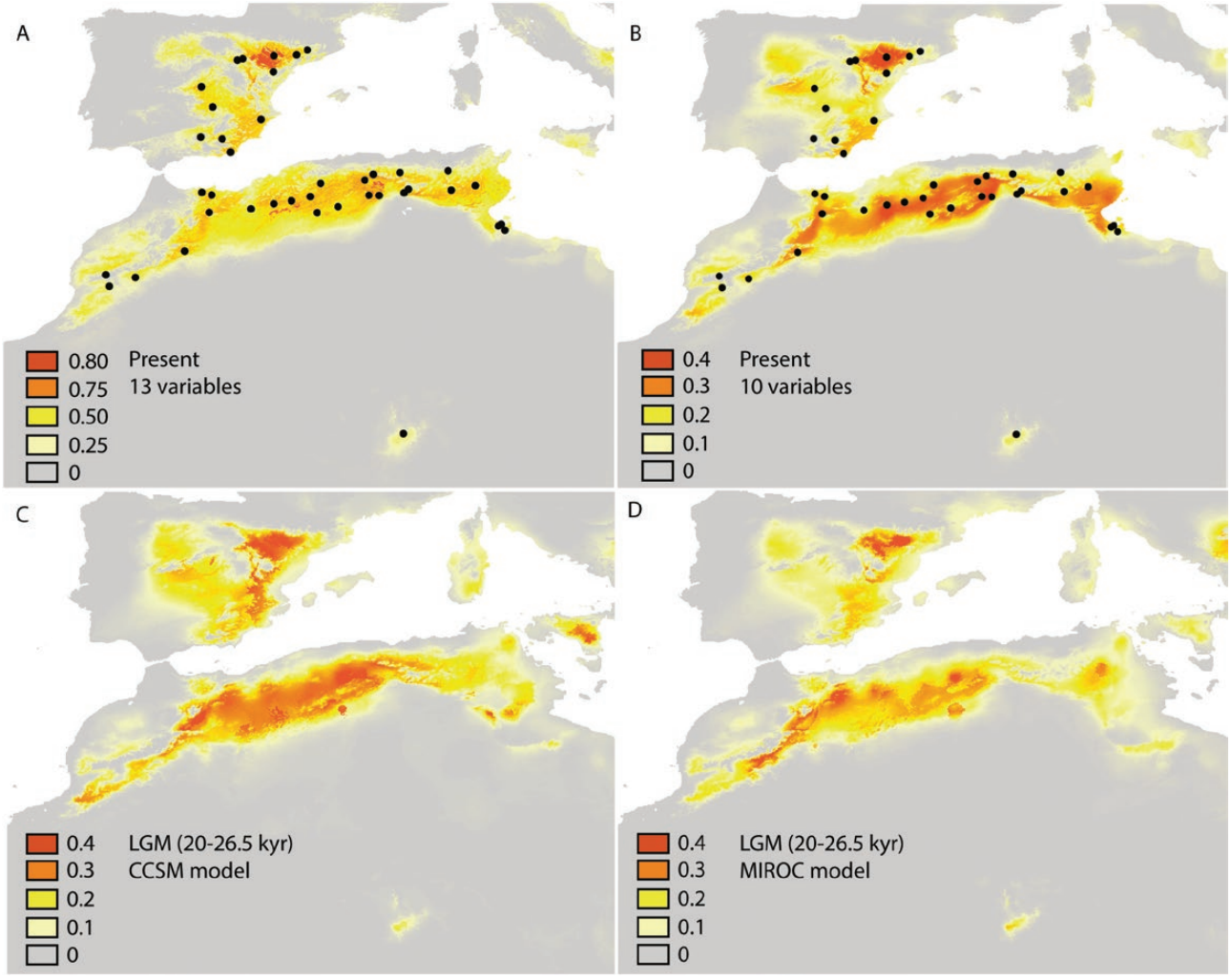

Figure 3. Potential distribution maps of *Artemisia herba-alba* obtained with MaxEnt under different modelling conditions: A, present model with 13 environmental variables; B, present model with ten environmental variables; C, Last Glacial Maximum CCSM model with ten environmental variables; D, Last Glacial Maximum MIROC model with ten environmental variables. Dots indicate sampled populations for this study.

the potential range distributions of the two cytotypes, when compared with their current occurrence in the studied area. The area under the curve values were high (> 0.80) in both cases (Supporting Information, Table S3), indicating a strong predictive power for the models (Loo, Mac Nally & Lake, 2007). Both models supported the occurrence of *A. herba-alba* in similar regions of the Iberian Peninsula and North Africa, but the total predicted area for tetraploid populations was lower (–55%) than for diploid populations (Table S3). Regarding the niche similarity between the cytotypes, the identity test revealed that the null distribution for indices *D* and *I* were not significantly larger ($P < 0.01$) than the observed values (Fig. 4C, D), indicating that the environmental niches are equivalent. Conversely, according to both estimated B1 and B2 metrics, niche breadth tests showed significant differences between ploidies (Fig. 4E, F), suggesting that the environmental niche of diploid cytotypes is larger than that of tetraploid cytotypes. PCA using the 13 environmental variables captured 75.2% of the variance in the first three components (PC1: 41.3%, PC2: 21.4%, PC3: 12.5%; Table S4). The 3D scatterplot for the first three components (Fig. S5), showing the ellipsoid that contains 50% of the data, showed that the environmental space of both cytotypes partially overlaps, with the niche of diploids being larger and containing that of the tetraploids. We did not find obvious environmental niche shifts between diploid and polyploid populations based on independent Wilcoxon signed-rank tests (Table S6; $P > 0.05$ in all cases). However, Levenne tests indicated that diploid

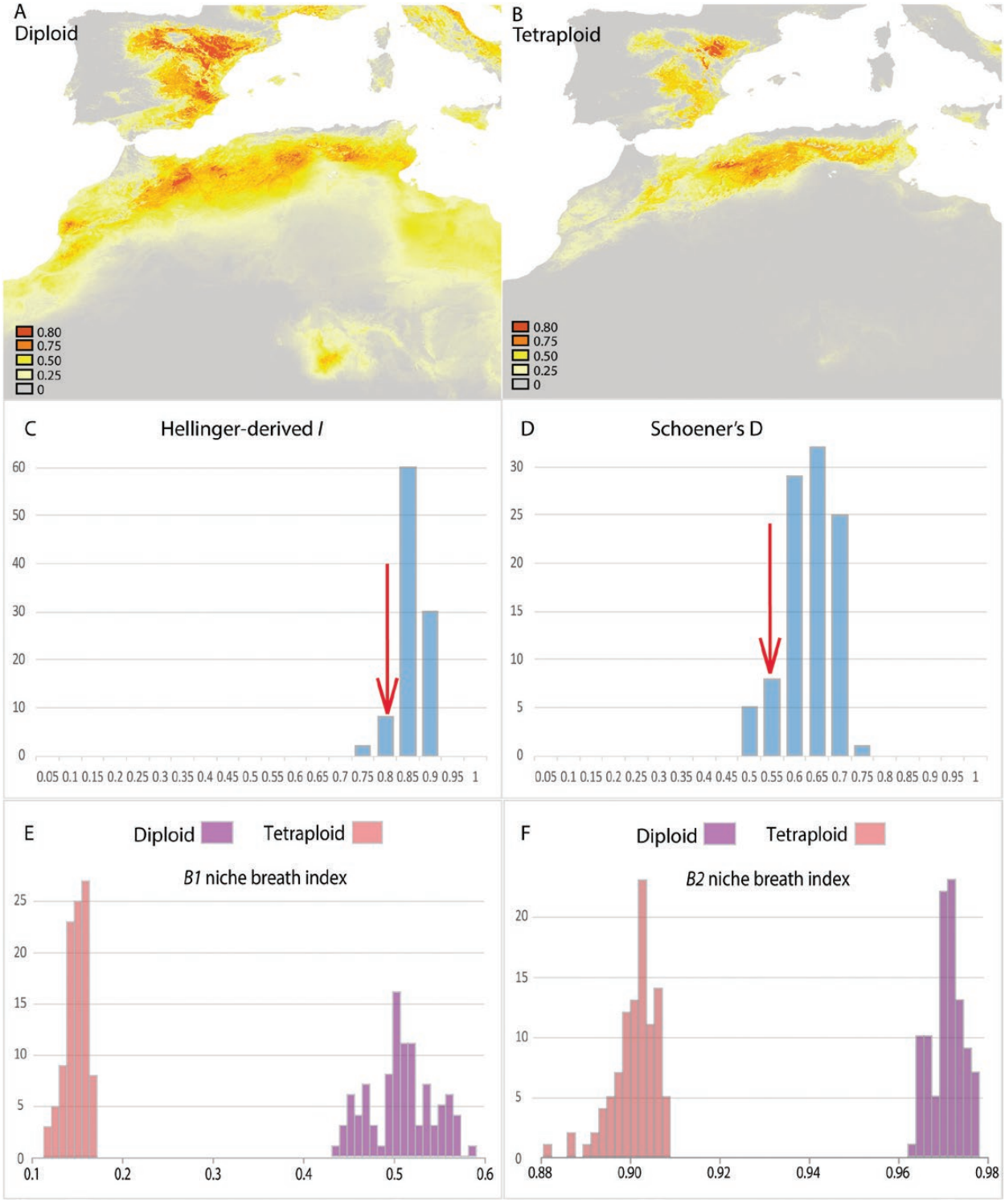

Figure 4. Niche comparison analyses between ploidies of *Artemisia herba-alba* populations. Independent environmental niche modelling for diploid (A) and tetraploid (B) populations of the species. Observed niche overlap values for the Hellinger-derived *I* index (C) and Schoener's *D* index (D) compared with a null distribution. In all cases, the similarity score (red arrow) is contained within the distribution predicted by the null hypothesis for niche equivalency, indicating that the environmental niches are equivalent. Niche breadth test according to the B1 index (E) and B2 index (F) indicating that the environmental niche of diploid cytotypes is significantly larger than that of tetraploid cytotypes.

populations show larger variances for bio12 (i.e. annual precipitation), organic content of the soil and pH of the soil than tetraploid populations ($P < 0.05$; see Table S6).

DISCUSSION

Comparably intricate phylogeographical patterns of *A. herba-alba* on both sides of the Strait of Gibraltar

Phylogeographical studies on plants distributed in both the northern and the southern parts of the Mediterranean Basin have frequently revealed European lineages to be nested in African clades and higher genetic diversity and differentiation at the intraspecific level in North Africa than in southern Europe (e.g. Guzmán & Vargas, 2009; Casimiro-Soriguer *et al.*, 2010; Veríssimo *et al.*, 2016; García-Aloy *et al.*, 2017; Bobo-Pinilla *et al.*, 2018; Villa-Machío *et al.*, 2018). Conversely, a few plant species occurring on both sides of the Strait of Gibraltar have shown the opposite pattern, i.e. the Iberian Peninsula is home to the most ancestral lineages and higher genetic diversity values (e.g. Escudero *et al.*, 2008; Jaramillo-Correa *et al.*, 2010). The complex phylogeographical structure we found in *A. herba-alba* does not clearly fit either of the two previous patterns. First, levels of haplotype and nucleotide diversity were similar between the Iberian Peninsula and North Africa (Table 2). Second, early-diverging haplotypes were observed in Iberian and North African populations (Fig. 2). Indeed, the evolutionary relationship between the haplotypes indicated the occurrence of several lineages on both sides of the Strait, independently of their phylogenetic distance (Fig. 1). Finally, partitioning schemes inferred by the spatial analyses of molecular variance explained a large proportion of the genetic variability of the species ($K = 2$, 59.94%; $K = 3$, 71.23%), but the biogeographical structure they showed was not related to the division of the Mediterranean Basin (Supporting Information, Fig. S4). Conversely, differentiation across the continents only accounted for 28.36% of the total molecular variance (Table S2), this result providing further evidence that the Strait is not a major phylogeographical barrier for this species.

To decipher the phylogeographical signals of a species we need to consider its biological features and how past climatic changes affected its particular distribution (Hewitt, 2004). Range expansions and contractions associated with Quaternary climatic oscillations are regularly related to extinction and recolonization processes, resulting in contrasting genetic patterns between refugia (e.g. higher diversity and endemism) and recently colonized regions (e.g. lower diversity and higher uniformity). In contrast, phylogeographical studies under climatic stability scenarios usually report high broad-scale diversity and spatial genetic complexity (Bilton *et al.*, 1998; Qu *et al.*, 2014; Faye *et al.*, 2016). Specifically, cold-tolerant taxa are inferred to have established widespread populations in continental lowlands of the Iberian Peninsula during full glacial stages, promoting the mixture of lineages or greater shifts in the spatial location of populations (Abellán & Svenning, 2014). ENM of *A. herba-alba*, which could be considered a chilling-tolerant species (Lyons, 1973) according to the values of minimal temperature of the coldest month (i.e. bio 6) in our studied localities, indicated the occurrence of similarly stable niches on the Iberian Peninsula and in North Africa under present and past LGM conditions (Fig. 3). Therefore, the intricate genetic structure of *A. herba-alba*, appearing unrelated to the geographical split established by the Strait of Gibraltar, fits the ecological stability inferred by our niche modelling results.

Despite the lack of a clear phylogeographical signal related to the division of the Mediterranean Basin, our analyses show certain genetic distinctiveness of the Algerian and Tunisian populations from the Moroccan and Iberian populations. Excluding the isolated Tamanrasset population, samples from Algeria to Tunisia have exclusive haplotypes (H1, H2, H3, H5, H6, H7 and H8) of this area (Fig. 1), and they only have one haplotype (P11, H4) which is shared with two populations of Morocco (P62 and P63). On the other hand, Morocco and the Iberian Peninsula share H16 between eight populations (P59, P64, P65, P66, P51, P52, P53 and P54). This east–west separation is also inferred in SAMOVA with $K = 2$ (Supporting Information, Fig. S4a), being also supported by Mantel tests showing significant spatial auto-correlation for North African populations but not for the whole dataset including populations from both continents. The Tamanrasset population (P60), which is approximately as distant from the rest of the Algerian populations as from the Moroccan populations, shares H19 with one population of Morocco (P66) and could be the result of a long-distance dispersal event. The Moroccan–Algerian phylogeographical split in *A. herba-alba* is consistent with some other intraspecific diversification studies on western Mediterranean plants (Terrab *et al.*, 2008; Naciri, Cavat & Jeanmonod, 2010; Taib *et al.*, 2020). This east–west disjunction in North Africa could be explained by a vicariance model with geographical breaks such as the Atlas range (e.g. Caujapé-Castells & Jansen, 2003; Andrés-Sánchez *et al.*, 2015) or the Rifan corridor crossed by the Moulouya river (e.g. Beddek *et al.*, 2018) having stronger effects as barriers than the Strait of Gibraltar. Alternatively, our results

could reflect ancient separate origins of the Moroccan and Algerian genetic pools (e.g. Magri *et al.*, 2007; Sánchez-Robles *et al.*, 2014). Solving this question would require molecular dating and biogeographical analyses that include other Mediterranean taxa closely related to *A. herba-alba*.

Besides the geological and climatic context during the evolutionary history of *A. herba-alba*, other biological characteristics probably played a role in shaping the phylogeographical patterns we have reported above. Most *Artemisia* spp. are wind-dispersed, and although achenes are lacking pappi, their relatively small size and light weight allow long-range dispersal to take place frequently (Laursen *et al.*, 2007). Species with propagules dispersed by wind have been reported to occur disproportionately on both sides of the Strait of Gibraltar (Lavergne, Hampe & Arroyo, 2013). *Artemisia herba-alba* is also an ecosystem dominant species, showing expansive distributions in dry steppes of the Iberian Peninsula and North Africa (Vallès, 1987; Le Houérou, 2001). Large population sizes, together with the stable habitats as suggested by our ENM results, have generally been associated with retention of ancestral polymorphisms (Schaal *et al.*, 1998). Indeed, the importance of incomplete lineage sorting mechanisms during the evolution of *Artemisia* subgenus *Seriphidium* has already been suggested by Malik *et al.* (2017). The complex mosaic-like haplotype distribution in *A. herba-alba*, in which some areas are dominated by certain lineages occasionally intermingled with other genetically unrelated haplotypes, could therefore be related to the dispersal and demographic characteristics of the species (for additional details see Vallès, 1989). Further studies using highly variable nuclear markers (e.g. microsatellite or next-generation sequencing approaches) would be necessary to confirm the role played by these and other factors in the evolutionary history of *A. herba-alba*.

Origins and persistence of polyploidy in *A. herba-alba*

A review of the available literature recently reported that geographical barriers seem to play a major role in driving the emergence and establishment of polyploid complexes in the Mediterranean flora (Marques *et al.*, 2018). In contrast, diploid and tetraploid populations of *A. herba-alba* were distributed without a clear geographical pattern, being equally well represented on both sides of the Strait of Gibraltar (Fig. 1). From a phylogeographical point of view, many haplotypes were found in both diploid and tetraploid populations, and evolutionarily early-diverging haplotypes were present in populations showing either of the two cytotypes (Fig. 2). Moreover, AMOVA results indicated that the ploidy of populations was not significantly associated with the genetic structure of the species (Supporting Information, Table S2). These results support the hypothesis that multiple events of whole genome duplication, giving rise to tetraploid populations from diploid populations, occurred during the evolutionary history of *A. herba-alba*, probably on both sides of the Mediterranean Sea.

Shared genetic background between co-occurring cytotypes is usually associated with repeated *in situ* formation of autopolyploids via unreduced gametes (Kolář *et al.*, 2017). Regarding *A. herba-alba*, autopolyploidy is also supported by morphological homogeneity (Vallès, 1987) as well as similar karyotypes (Vallès & Siljak-Yakovlev, 1997) between diploid and tetraploid cytotypes. Recurrent autopolyploidization events have been inferred in many other plant species showing similar combinations of phylogeographical and cytogeographical patterns (e.g. Segraves *et al.*, 1999; Yamane, Yasui & Ohnishi, 2003; Mairal *et al.*, 2018). Specifically, multiple origins of autopolyploids have also been reported in *Artemisia tridentata* Nutt. (Richardson *et al.*, 2012), an evolutionarily distant congener showing noticeable ecological parallelisms with the species studied here. The alternative hypothesis of frequent crossing between diploid and tetraploid genotypes as a source of haplotype diversity in *A. herba-alba* tetraploids is unlikely due to the lack of triploid genome size assessments in our data [intermediate ploidy (triploids, 3*x*) would be expected as a mediator of gene flow (Kolář *et al.*, 2017)]. The vast majority of populations we studied (all except two) exclusively showed either diploid or tetraploid individuals, suggesting the existence of reproductive barriers between the two cytotypes and the occurrence of frequency-dependent exclusion by minority cytotype disadvantage (Levin, 1975; Husband, 2000).

The recurrent origins of autotetraploids, together with the high production of anemochorous dispersed achenes of *A. herba-alba* (Vallès, 1989), could explain the broad occurrence of both diploid and polyploid cytotypes across the distribution range of the species, but it is not sufficient to explain their maintenance. Under a scenario of minority disadvantage, newly originating cytotypes would experience frequency-dependent selection and they would be excluded by drift from the population of the progenitors in a few generations (Levin, 1975). Therefore, changes in environmental requirements, promoting eco-spatial segregation and within-cytotype mating, would be necessary for the establishment and persistence of populations with different ploidies (Felber, 1991). The presence of within-cytotype gene flow in *A. herba-alba* is suggested by the frequency of diploid and tetraploid populations showing more than one haplotype (Fig. 1). Regarding the ecological differentiation, our modelling

comparisons did not support a significant shift in the environmental niche of either cytotype, but the analyses revealed a significant variation in their niche breadths (Fig. 4). Despite the similarly wide range of distribution of both cytotypes, a larger environmental space in diploid than in tetraploid populations was also inferred by their predicted potential areas (Supporting Information, Table S3). Finally, PCA results illustrate that the tetraploid niche is fully nested in the niche breadth of diploid populations (Fig. S5).

Niche comparisons between cytotypes in which the niche of one ploidy is narrower and nested in the niche of the other cytotype have been interpreted as indicating environmental specialization (e.g. Parisod & Broennimann, 2016; Castro *et al.*, 2019). Therefore, to establish successfully, tetraploids of *A. herba-alba* could be thriving in particular areas of the landscape where they would outcompete the progenitor diploids and, thus, avoid the minority cytotype exclusion. According to our statistical tests to explore the environmental variable differences between ploidies, tetraploid populations specifically showed narrower variance for annual precipitation, organic content of the soil and pH of the soil (Supporting Information, Fig. S6). In several plant species, autotetraploids show a tendency to occupy ruderal and more disturbed habitats, whereas diploids are not so restricted (e.g. Španiel *et al.*, 2008; Rivero-Guerra, 2008; Kolář *et al.*, 2016; Castro *et al.*, 2019). The narrower variance shown by tetraploid populations of *A. herba-alba* for the two studied soil variables, particularly for the organic content, could indicate this specialization in disturbed habitats. However, to test this hypothesis, reciprocal transplant experiments would be necessary to confirm that tetraploids are more or less able to develop than diploid under certain environmental conditions.

CONCLUSIONS

To our knowledge, this is the first study combining DNA sequencing, genome size assessments and niche modelling on a plant species widely distributed on both sides of the Mediterranean Sea. Such an integrative approach enabled the inference of various unexpected phylogeographical and cytogeographical patterns. Our results suggest that the Strait of Gibraltar did not play a major role in shaping the genetic diversity and structure of *A. herba-alba*, and we did not find evidence of any particular area acting as a genetic reservoir or refugium for this species. Likewise, multiple polyploidization events were inferred to have occurred in different regions during the evolutionary history of the plant. According to our environmental modelling analyses, palaeoecological stability, together with the dispersal and demographic characteristics of the species, was hypothesized as a potential driver of the intricate geographical distribution of genotypes and cytotypes reported here. The environmental comparisons among cytotypes indicated that polyploidization did not cause a significant shift in the niche of *A. herba-alba*, but tetraploids showed narrower ecological preferences that could explain their frequent and successful establishment. Overall, as was suggested for the evolution of *Artemisia* subgenus *Seriphidium* (Malik *et al.*, 2017), our study highlights a complex natural history that underlies the morphological uniformity in this key species of the Mediterranean dry steppes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Table S1. Geographical information, GenBank accession numbers, herbarium vouchers, ploidy levels and nuclear DNA amount data of the studied populations.

Table S2. Genetic variability values in the geographical and cytogenetical groups of populations defined in the study.

Table S3. Predicted potential distribution of *Artemisia herba-alba* under different models, with area comparison and performance details.

Table S4. Component loadings of the different variables and the relative importance of the first three components.

Table S5. Median and variance comparisons among diploid and tetraploid *Artemisia herba-alba* populations for the environmental variables included in the study.

Fig. S1. TCS network representing the haplotypes of *Artemisia herba-alba* with the colours indicating geographical distribution. Black stripes represent unsampled intermediate haplotypes, one base mutation distant. The size of the circles represents the number of individuals.

Fig. S2. Parsimony network representing the haplotypes of *Artemisia herba-alba* with the colours indicating cytotype distribution. Black stripes represent unsampled intermediate haplotypes, one base mutation distant. The size of the circles represents the number of individuals.

Fig. S3. Values of ΔF_{CT} used to estimate the most likely K from SAMOVAs.

Fig. S4. Geographical distribution of the populations according to the spatial genetic partitioning defined by SAMOVA: A, $K = 2$ groups; B, $K = 3$ groups.

Fig. S5. 3D scatterplot of the first three axes from the principal component analysis (PCA) for the 39 populations studied of *Artemisia herba-alba*. The ellipsoids represent the space containing 50% of the data from diploid (blue) and tetraploid (pink) populations.

Fig. S6. Boxplots representing values of the environmental variables, as well as PCA values, for the studied populations of *Artemisia herba-alba* according to their different ploidy level